Comparisons of extraction and purification methods of soil microorganism DNA from rhizosphere soil

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Abstract: Microorganism DNA of rhizosphere soil from *Pinus koraiensis* and *Pinus sylvestriformis* were extracted by proteinase K based on SDS method, CTAB method, PVP (polyvinylpolypyrrolidone) method, and freezing and thawing method and the crude DNA from rhizosphere soil were purified by dialysis method, silver beads absorption method, and squeezing DNA gel method. The results of different extracting and purifying methods were compared and evaluated. Results indicated that the best method of extraction for microorganism DNA in rhizosphere soil was proteinse K based on SDS method with high salt concentration of 1.0% (w/v) NaCl, which could effectively eliminate humic acids and other impurities. The dialysis method was suitable to purify DNA from rhizosphere soil because of effectively removing brown matters and humic acids and the purified products were suited to PCR amplification. Squeezing DNA gel method was also a good purification method with the advantage of inexpensive in cost and efficient in use.

Keywords: Extraction; Microorganism DNA; *Pinus koraiensis*; *Pinus sylvestriformis*; Purification; Rhizosphere soil
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Introduction

Molecule techniques based on nucleic acid have led to tremendous changes in detecting natural environmental microorganism (Liesack *et al*. 1992; Ward *et al*. 1990). In the past, a cultivation dependent approach only can examine 0.1%−1% of total environmental microorganism specially for soil (Amann *et al*. 1995; Brock 1987; Haws 1991; Luo *et al*. 2003; Ma *et al*. 2003), and this result hardly indicate comprehensive profile of soil microorganism diversity in situ (Amann *et al*. 1995; Luo *et al*. 2003). Since molecule techniques based on nucleic acid were introduced to determine soil microorganism, some great changes had happened in understanding the structure and the dynamics of soil microbial communities and gave access to previously unknown parts of soil microbial diversity (Lionet *et al*. 2003). Though these molecular approaches circumvent limitations due to the selectivity and unrepresentative nature of culture-based methods, several biases generated in the DNA extraction and purification procedure weaken the robustness of the analysis (Head *et al*. 1998). Laurent *et al*. (2001) considered that the recovery of microbe diversity was influenced by the DNA extraction method from soil. The purity of the DNA from soil was often unsatisfactory, particularly in the case of soils rich in humic compounds (Courtois *et al*. 2001) such as rhizosphere soil of plant. And extraction of DNA from soils always results in coextraction of humic substances which can interfere with DNA detection and measurement (Zhou *et al*. 1996) and purification. This contamination can inhibit the activity of *Taq* polymerase in PCR and lead to no product in PCR amplification (Luo *et al*. 2003; Tsai *et al*. 1996). Especially for rhizosphere soil, contaminations of plenty of high-molecule humic acids, exudations from roots, and other high-molecule compounds decomposed from plant root residues are very crucial problems at the processes of PCR amplification (Smalla *et al*. 1993). Therefore, the selection of extraction and purification methods is a very crucial for DNA of rhizosphere soil microorganism.

The aim of this study is to compare and evaluate the different methods of DNA extraction and purification from rhizosphere soils through analyzing simplicity, purity, and yields of DNA for rhizosphere soil samples of *Pinus koraiensis* and *Pinus sylvestriformis* seedlings.

Materials and methods

Sample collection

Rhizosphere soils were collected from the fine roots of *P. koraiensis* and *P. sylvestriformis* seedlings in growing season in Changbai mountain located in the northeast of China (128° 28′E, 42° 24′N; 738 m a.s.l). The properties of bulk soil texture were shown in Table 1. Fine roots at the depth of 0–10 cm were severed and brought to lab. Excess bulk soils were flaked away and those attached to roots were rhizosphere soils (Smalla *et al*. 2001). Then the rhizosphere soils were washed off with sterile 0.85% NaCl solution (Schmalenberger *et al*. 2003) and put in a sterile bucker. The resulting soil slurry was centrifuged at 3 000 $r \cdot min^{-1}$ for 20 min to wipe off the water from the rhizopshere soil. The rhizosphere soil was stored at -20 ºC for analysis.

Extraction of soil DNA

Proteinase K based on SDS method: Rhizosphere soil (0.5 g) was extracted with 1.5 mL lysis buffer (100 $mmol \cdot L^{-1}$ tris-HCl (pH 8.0), 100 $mmol \cdot L^{-1}$ phosphate (pH 8.0), 100 $mmol \cdot L^{-1}$ sodium EDTA (pH 8.0), 1% CTAB (Hexadecylmethylammonium bromide), 1.5 $mol \cdot L^{-1}$ NaCl) (EllingsØe *et al*. 2002; England *et al*. 2001) and 15 μL proteinase K (10 $mg \cdot mL^{-1}$) through horizontal shaking at 225 $r \cdot min^{-1}$ for 30 min at 37ºC (Zhou *et al*. 1996; Zhang *et al*. 2003). Then, 160 μL of 20% SDS was added and the

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samples were incubated in a 65ºC bath for 2 h with gentle end over end inversion every 15–20 min. The supernatant was collected after centrifuging at 6 000 r·min^{-1} for 10 min at room temperature and translated to 1.5 mL eppendorf tube. The supernatant was mixed with an equal volume phenol-chloroform-isoamyl alcohol (25:24:1) to remove protein and amylose. The aqueous phase was recovered by centrifugation at 6 000 r·min^{-1} for 10 min and precipitated with 0.6 volume isopropanol at 4ºC overnight. The crude nucleic acids was obtained by centrifugation at 6 000 r·min^{-1} for 15 min at 4ºC (eppendorf centrifuge 5417 R, made in Germany) and the precipitation was washed with cold 70% ethanol and re-suspended in TE buffer (10 mmol·L^{-1} Tris-HCl, 1 mmol·L^{-1} EDTA, pH 8.0) (Tesar *et al*. 2002) up to final volume of 50 μL (Chen *et al*. 2002).

Table 1. The properties of bulk soil related to rhizosphere soil

Plant types	Soil texture	Moisture content % (w/w)	pH	$C_{org.}$ (g·kg^{-1})	N (g·kg^{-1})
Pinus koraiensis	Dark brown soil	21.4±0.09	6.3±0.13	5.29±0.52	0.34±0.03
Pinus sylvestriformis	Dark brown soil	15.4±0.41	6.5±0.25	4.13±0.27	0.26±0.05

CTAB method: Rhizosphere soil (0.5 g) was extracted with 1.5 mL lysis buffer (100 mmol·L^{-1} tris-HCl (pH 8.0), 100 mmol·L^{-1} phosphate (pH 8.0), 100 mmol·L^{-1} sodium EDTA (pH 8.0), 2% CTAB (Hexadecylmethylammonium bromide), 1.5 mol·L^{-1} NaCl) (EllingsØe *et al*. 2002), then, 160 μL of 20% SDS was added. The samples were incubated in a 65℃ bath for 2 h with gentle end over end inversion every 15–20 min. The rest procedures were the same with the first method.

PVP (Polyvinylpolypyrrolidone) method: Rhizosphere soil (0.5 g) was extracted with 1.5 mL lysis buffer (50 mmol·L^{-1} tris-HCl (pH 7.0), 20 mmol·L^{-1} sodium EDTA (pH 7.0), 1.2% PVP (Polyvinylpolypyrrolidone), 20% SDS), and the samples were incubated in a 65℃ bath for 0.5 h with gentle end over end inversion every 15–20 min. After the supernatant was purified with an equal volume phenol-chloroform-isoamyl alcohol (25:24:1), 1/10 volume NaAc (0.1 mol·L^{-1}, pH 5.2) and 0.6 volume isopropanol were added to it and was deposited at 4℃ for overnight. The rest procedures were the same with the first method.

Freezing and thawing method: 0.5 g (EllingsØe *et al*. 2002) rhizosphere soil samples added extraction buffer (100 mmol·L^{-1} tris-HCl (pH 8.0), 100 mmol·L^{-1} phosphate (pH 8.0), 100 mmol·L^{-1} sodium EDTA (pH 8.0), 1% CTAB (Hexadecylmethylam-monium bromide), 1.5 mol·L^{-1} NaCl) were frozen and thawed at -65ºC–65ºC for three cycles for 30 min every times. The rest procedures were the same with the first method.

DNA purification

On the base of the DNA extraction methods, the DNA of rhizosphere soil was purified by using the following three methods.

Dialysis method: We designed a purification set (Fig. 1). The electrophoresis (50V) was about 30 min and the direction was from DNA gel piece which was excised from the agarose electrophoresis to dialysis membrane. Inverted electrophoresis was about 20 s after the DNA was thoroughly released into TAE buffer (300 μL or so). After the electrophoresis, acetic ammonium (2.5 mol·L^{-1}, pH 6.1) was added and the TAE buffer between 0.6% agarose gel and the dialysis film was precipitated with cold ethanol for 2 h at 4ºC. The precipitation was dissolved in TE buffer (pH 8.0).

Silver beads absorption method: DNA was purified by using DNA gel purification kit SK1111 (made in Songon Corporation in Shanghai in China).

Squeezing DNA gel method: The crude DNA was isolated from the agarose electrophoresis gel by squeezing the DNA gel pieces frozen at -20ºC overnight. The freezing DNA gel pieces were immediately squeezed by fingers after wrapped in a piece of sterile Parafilm. Furthermore, the buffer squeezed out from the DNA agarose gel pieces was transferred by tip to a 1.5 mL Eppendorf tube, which could be used in other determinations such as PCR amplification and DNA hybridization.

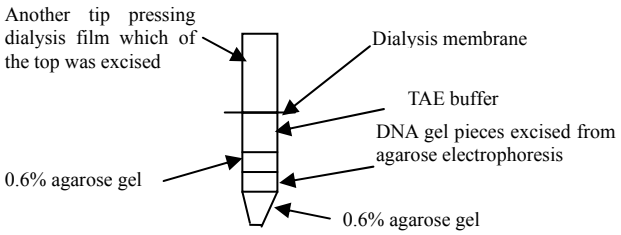

Fig.1 The dialysis set of DNA

Evaluation of DNA quality

The quality of DNA was evaluated by determining the absorption of nucleic acid at λ_{260} by Eppendorf Bio-photometer, the values of A_{260} /A_{280} and A_{260}/A_{230}, DNA gel electrophoresis, and PCR amplification of 16s rDNA.

PCR amplification

Each PCR mixture (50 μL) was composed of 10 ×*Ex Taq* buffer (Mg^{2+} plus), dNTP mixture (2.5 mmol·L^{-1}), *TaKaRa Ex Taq* (5 U·μL^{-1}), template DNA (10 ng), primer 341F (5'-CCTAC GGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTMTTTGA GTTT - 3') (30 pmol, respectively), and appropriate sterile deionized water up to 50 μL. Program of PCR amplification consisted of one cycle of 95 ºC for 5 min; 35 cycles of 94 ºC for 2 min, 60 ºC for 1 min, 72 ºC for 1 min, and finally one cycle of 72 ºC for 10 min. The amplification products were determined by gel electrophoresis in 1% agarose. And the gel images were acquired by the ChemDoc (Bio-Rad) Gel Documentation System.

Results and discussion

Comparison of DNA extraction methods

Of these four extraction methods, the proteinase K SDS-based method was the best one and this result could be obtained by determining the OD value of the DNA at 260 nm. Result showed that both yields and purity of DNA extracted by the proteinase K SDS-based method were higher than those by other three methods (Table 2) and the size of fragment was 23 kb. CTAB method did not decrease the DNA yields extracted from rhizosphere soils and could effectively remove humic materials, but the ratios of A_{260} / A_{280} and A_{260} / A_{230} were lower. PVP method, which could also remove amount of humic materials, could bind DNA closely so as to lead to the decrease of the DNA yields (Zhou *et al*. 1996). Freezing and thawing method was the one without severe shear-

ing to DNA, but the DNA yield was lower than others (Fig. 2).

Table 2. DNA yields and purities of the crude DNA from rhizosphere soil by different treatments

Rhizosphere soil types	DNA extracting methods	DNA yields (μg·g^{-1} (dry wt) of soil)	A_{260} / A_{280} ratio	A_{260} / A_{230} ratio
Pinus koraiensis	Proteinase K based on SDS	53.9±0.18	1.53±0.04	1.61±0.01
	1% CTAB	51.6±1.01	1.14±0.01	0.89±0.02
	PVP.	42.7±0.36	1.17±0.05	1.23±0.06
	Freezing and thawing	49.7±1.22	1.14±0.02	0.91±0.03
Pinus sylvestriformis	Proteinase K based on SDS	65.38±0.21	1.09±0.07	1.21±0.02
	1% CTAB	61.29±0.31	1.11±0.03	1.07±0.05
	PVP.	56.80±0.16	1.21±0.04	0.98±0.02
	Freezing and thawing	58.33±0.24	1.01±0.01	0.87±0.05

Notes: DNA yields (mean values, n = 5, ± represents standard deviation) were calculated by the OD value of DNA determined by Eppendorf Bio-photometer. The ratios of A_{260} / A_{280} and A_{260} / A_{230} were displayed when the OD value of DNA was determined.

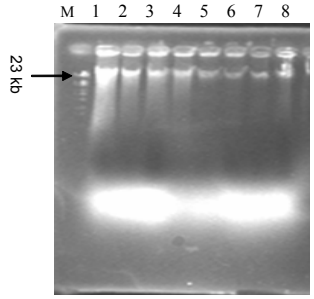

Fig. 2 The electrophoresis results of different extraction methods [a)]

Notes: Lanes from M to 8 were the marker λ-EcoT14 I fragment, crude DNA solutions extracted by proteinase K method (lanes from 1 to 2), by CTAB method (lanes from 3 to 4), by PVP method (lanes from 5 to 6), and by freezing and thawing method (lanes from 7 to 8), respectively. The lanes 1, 3, 5, and 7 were from the rhizosphere soil of *Pinus koraiensis* and the others were from *Pinus sylvestriformis*.

The reason for the proteinase K extraction based on SDS method had best extracting effects is that proteinase K could effectively break up the cell wall of microorganism to release DNA easily (Zhang *et al*. 2003). The methods of CTAB and PVP could effectively remove the contaminations of high-molecule humic acids. However, the use of PVP always decreases the DNA yields because of its binding DNA closely (Zhou *et al*. 1996). And the low DNA yields influence the analysis for diversity of soil microorganism. Although CTAB method could not decrease the DNA yields, the function of DNA was affected by high salt concentration. Liesack *et al* (1992) and Ogram *et al*. (1987) considered that bead mill homogenization and other physical methods such as sonication generally cause severe DNA shearing. However, Zhou *et al*. (1996) reported that the combination of grinding, freezing-thawing, and SDS methods could gave much higher DNA yields without severe shearing.

DNA purification and evaluation of DNA quality

DNA from rhizosphere soils was extracted by Proteinase K based on SDS method and was purified by using the above three methods. Results showed that the dialysis method was the best purification method that could be proved from the electrophoresis results (Fig. 3). The squeezing DNA gel method was also a good purification method because it is convenient in use and inexpensive in cost. The purification efficiency by silver beads absorption was the lowest among these methods, which was possibly associated with the high loss of the absorption of silver beads towards DNA. Plenty of high-molecule-humic and other impurities might be also absorbed in silver beads, thus leading to low absorption efficiency towards DNA (Fig. 4). Furthermore, the loss of silver beads purification was so high that the OD value of DNA could not be determined (Table 3). This may decrease the detection efficiency of diversity of environmental microbial samples (EllingsØe *et al*. 2002)

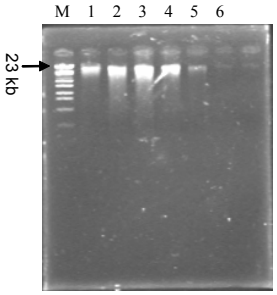

Fig.3 Electrophoresis results from different purification methods

Notes: Lane M was the marker λ-EcoT14 fragment, lanes 1 and 2 were squeezing DNA gel pieces, 3 and 4 were the dialysis method, and 5 and 6 were silver beads absorption method, respectively. Here lanes 1, 3, and 5 were the rhizosphere soil from *Pinus koraiensis* and others were from *Pinus sylvestriformis*.

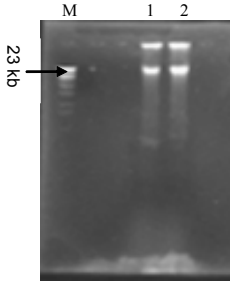

Fig.4 Electrophoresis results of the solutions abandoned after purified by the silver beads

Notes: Lanes 1 and 2 were *Pinus koraiensis* and *Pinus sylvestriformis* rhizosphere soil, respectively. The marker was λ-EcoT14 I fragment and the largest size of fragment was 23 kb.

Table 3. DNA yields and purities of the purified DNA by different purification treatments

Rhizosphere soil types	Treatments	DNA yields (μg·g^{-1} (dry wt) of soil)	A_{260} / A_{280} ratio	A_{260} / A_{230} ratio
Pinus koraiensis	Dialysis method	35.20±0.09	1.79±0.03	2.03±0.09
	Squeezing DNA gel method	30.40±0.15	1.72±0.07	1.96±0.05
	Silver beads	--------	--------	--------
Pinus sylvestriformis	Dialysis method	42.19±0.04	1.75±0.06	2.11±0.02
	Squeezing DNA gel method	39.26±0.06	1.74±0.02	2.04±0.03
	Silver beads	--------	--------	--------

Notes: Sample purified was crude DNA extracted through proteinase K based on SDS method.

The results from Table 2 indicated that the contamination of humic and protein were very severe for crude DNA. After crude DNA was purified through the dialysis method, contaminations were basically eliminated (Table 3). And the PCR amplification

results were satisfying except for using the template which the silver beads purified (Fig. 5).

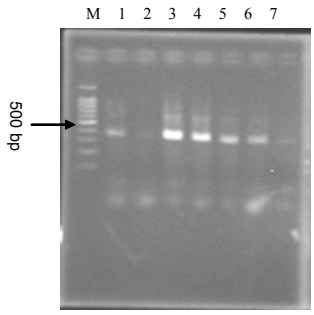

Fig. 5 1% agarose electrophoresis results of PCR products by using different templates DNA purified through different methods

Notes: M was the 100 bp ladder marker, lane 7 was the PCR product of the control, and lanes from 1 to 2, 3 to 4, 5 to 6 were the PCR products of different templates DNA purified through silver beads purification, dialysis method, and squeezing DNA gel method, respectively. And lanes 1, 3, and 5 were the rhizosphere soil from *Pinus koraiensis* and 2, 4, and 6 were from *Pinus sylvestriformis* rhizosphere soil.

The dialysis and squeezing DNA gel methods were advisable for PCR amplification and their costs were also very low. The dialysis method was suitable to purify DNA from rhizosphere soil and could thoroughly remove brown matter from the crude DNA solution, although the operation was relatively complicated. Furthermore, the squeezing DNA gel method was also suitable to purify DNA extracted from rhizosphere soil and it was the most inexpensive and simplest method.

Conclusions

In summary, the methods of DNA extraction and purification of soil microorganism from rhizosphere soils were evaluated. The most suitable method for DNA extraction of soil microorganism from rhizosphere soil samples of *P. koraiensis* and *P. sylvestriformis* was proteinase K SDS-based method. Dialysis method was suitable to purify DNA extracted from rhizosphere soils and could thoroughly remove brown matter from the crude DNA solution, although the operation was relatively complicated. Moreover, the squeezing DNA gel method was also a good method and the yield and the purity were suitable to latter operation such as PCR amplification. We considered that silver beads absorption purification because of its high loss ratio may lead to the decrease of microbial diversity of environmental samples analyzed, which can be detected by DGGE (denaturing gradient gel electrophoresis) (Schmalenberger *et al*. 2003; Brock, 1987; Fujimoto *et al*. 2003). It is important to recognize that no single method of cell lysis or purification will be appropriate for all rhizosphere soils and experimental goals. The basic methods suggested should be appropriate for the more common cases, but different combinations and modifications of lysis and purification protocols will probably be needed for some conditions (Zhou *et al*. 1996).

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